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16. ABSTRACT

The influence of nitrogen dioxide (NO_2) on pentobarbital(PEN)-induced sleeping time(S.T.) was investigated in mice. Acute exposure to concentrations as low as 470 ug NO_2/m^3 (0.25 ppm) caused a significant increase in PEN-induced S.T. No significant effects on PEN-induced S.T. were detected after 1 or 2 days (3 h/day) exposure to 235 ug NO_2/m^3 (0.125 ppm). None of the exposure regimens affected the time required for the drug to induce sleep. When the effects of repeated daily exposures (3 h/day) to 9400 ug NO_2/m^3 (5 ppm) were compared in male and female mice, the females had a significantly increased PEN-induced S.T. after 1 and 2 days of exposure. However, an increase in PEN-induced S.T. was not observed in males until the 3rd day of exposure. Since the duration of PEN-induced S.T. is primarily determined by hepatic mixed function oxidase activity, it is possible that NO_2 may alter some aspects of xenobiotic metabolism.

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EXTRAPULMONARY EFFECTS OF NO_2 AS REFLECTED BY PENTOBARBITAL-INDUCED SLEEPING TIME IN MICE

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SUMMARY

The influence of nitrogen dioxide (NO_2) on pentobarbital (PEN)-induced sleeping time (S.T.) was investigated in mice. Acute exposure to concentrations as low as 470 $\mu g\ NO_2/m^3$ (0.25 ppm) caused a significant increase in PEN-induced S.T. No significant effects on PEN-induced S.T. were detected after 1 or 2 days (3 h/day) exposure to 235 $\mu g\ NO_2/m^3$ (0.125 ppm). None of the exposure regimens affected the time required for the drug to induce sleep. When the effects of repeated daily exposures (3 h/day) to 9400 $\mu g\ NO_2/m^3$ (5 ppm) were compared in male and female mice, the females had a significantly increased PEN-induced S.T. after 1 and 2 days of exposure. However, an increase in PEN-induced S.T. was not observed in males until the 3rd day of exposure. Since the duration of PEN-induced S.T. is primarily determined by hepatic mixed function oxidase activity, it is possible that NO_2 may alter some aspects of xenobiotic metabolism.

INTRODUCTION

The effects of NO_2 are well documented [12]. Such effects include: morphological alterations (including emphysema-like lesions), increased susceptibility to infectious respiratory disease, dysfunction of pulmonary mechanics, and changes in concentrations and activities of antioxidant

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Abbreviations: I.T., induction time; PEN, pentobarbital; S.T., sleeping time.

enzymes and altered lipid patterns. Studies of extrapulmonary effects have been limited. To improve understanding of the overall health effects of NO_2, more investigations of the potential NO_2-induced systemic toxicity are needed.

The types of effects described above for NO_2 have also been observed following exposure to ozone (O_3) [20]. Where comparisons are possible, O_3 is usually effective at a lower concentration and thus is thought to be more toxic. The similarity of effects may be a result of the potential of each gas to produce free radicals and other potent oxidizing species. Therefore, when O_3 was found to cause a series of effects which can be interpreted as effects on xenobiotic metabolism of the liver [6, 8] similar studies were initiated with NO_2. The research reported here is the first phase of studies in which the influence of various concentrations and times of exposure to NO_2 on PEN-induced S.T. was determined. Since the drug-induced S.T. model is accepted as in vivo assessment of xenobiotic metabolism, this model was utilized to obtain concentration and time data to provide direction for future mechanistic studies.

MATERIALS AND METHODS

Animal exposures

Male and female, specific pathogen-free, CD-1 mice (COBS, Charles River Breeding Laboratory, Wilmington, MA), weighing 18—22 g (4—6 weeks old) were used. Mice were maintained on a bedding of white pine shavings until use (between 6—11 days after receipt) and received food (Wayne LabBlox) and water ad libitum. A 12 h photoperiod was maintained.

Animals were placed in individual stainless steel wire cages for exposure within a 0.32 m^3, Rochester-type chamber, as described previously [8]. Nitrogen dioxide (0.1% NO_2 in nitrogen) was diluted with filtered room air and introduced into the exposure chamber at a rate of 1 air change/min. Nitrogen dioxide concentrations were monitored continuously with a chemiluminescence NO_2 analyzer (Bendix, Lewisburg, WV). The NO_2 monitor was calibrated using the gas phase titration technique [19]. Control animals were exposed to filtered room air in an identical type chamber as that used for NO_2 exposure. All exposures began at about 9 am and were for 3 h/day.

Measurement of PEN-induced S.T.

To reduce the influence of circadian rhythm and room temperature on the results [4, 13, 17] all PEN-induced S.T. studies were begun at about 1:00 pm immediately after NO_2 or air exposure and were conducted in an environmentally controlled room with a temperature of 24° ± 1°C and a relative humidity of 46% ± 2%. The method of Gardner et al. [6] was used for the PEN-induced S.T. measurements. Mice were injected intramuscularly with 50 mg/kg PEN in an approximate volume of 0.2 ml. The PEN solution

was a saline dilution of Nembutal (Abbott Laboratories, Stone Mountain, GA). Both I.T. (time elapsed between injection and the loss of righting reflex i.e., the animal remaining on its back) and S.T. (the length of time elapsed between the loss and regaining of the righting reflex) were measured.

Experimental design and statistical methodology

On any given day, groups of mice were exposed to either air or NO_2. For each concentration of NO_2, the effects on drug-induced I.T. and S.T. of various lengths of exposure to the pollutant were analyzed using two-way analysis of variance. Then Dunnett's test [5] was used to determine the pattern of significant treatment differences.

RESULTS

Initial studies were conducted with male and female mice exposed to 9400 μg NO_2/m^3 (5 ppm) for 3 h/day. In females there was a statistically significant increase in PEN-induced S.T. following 1 and 2, but not 3, days of NO_2 exposure (Table I). An increase ($P < 0.05$) in PEN-induced S.T. did

TABLE I

EFFECT OF REPEATED (3 h/DAY) EXPOSURE TO NO_2 ON PENTOBARBITAL-INDUCED SLEEPING TIME IN FEMALE MICE

ppm NO_2	Number of daily exposures	n[a]	Sleeping time, min.	
			Air	NO_2
5.0	1	59	21.4 ± 0.4[b]	28.9 ± 1.2[c]
	2	45	24.7 ± 0.9	27.5 ± 1.2[c]
	3	30	21.9 ± 0.8	23.2 ± 1.4
1.0	1	43	21.8 ± 0.6	29.5 ± 0.9[c]
	2	45	21.8 ± 0.7	24.9 ± 1.1[c]
	3	43	23.0 ± 0.8	17.3 ± 0.8[c]
	4	29	21.3 ± 0.7	20.8 ± 0.9
0.5	1	29—30	25.8 ± 0.9	26.6 ± 1.3
	2	28—29	21.6 ± 0.9	30.6 ± 1.6[c]
	3	15	21.6 ± 0.8	19.1 ± 1.1
0.25	1	56—57	20.6 ± 0.8	24.4 ± 1.5[c]
	2	56—58	23.0 ± 0.8	22.6 ± 1.1
	3	37—43	17.4 ± 1.2	16.9 ± 1.0
0.125	1	60	31.8 ± 2.0	29.4 ± 1.8
	2	58—59	25.6 ± 1.1	26.0 ± 1.2

[a] n, number of mice/exposure group.
[b] All data are means ± SE.
[c] Different from the corresponding air group ($P < 0.05$).

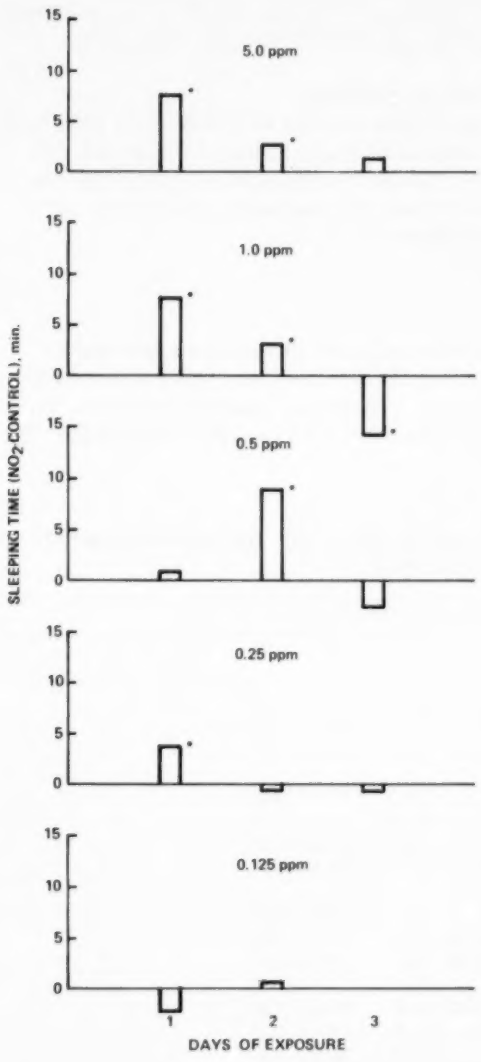

Fig. 1. Effect of repeated daily (3 h/day) exposures to NO_2 on pentobarbital-induced sleeping time in female mice. * indicates that the mean response of the NO_2 group was different ($P < 0.05$) from the mean air response. Sample sizes and SE are given in Table I.

not occur in male mice until after 3 days of NO_2 exposure (26.5 min ± 1.6 SE vs. 33.5 min ± 2.1 SE). For both male and female mice there were no statistically significant effects of 9400 μg NO_2/m^3 (5 ppm) on PEN-induced I.T. Since for acute exposures female mice were more sensitive than male mice to NO_2 exposure, concentration-response studies with lower NO_2 levels were conducted using female mice (Table I).

The effects of 1 and 2 daily (3 h/day) exposures to 9400 μg NO_2/m^3 (5 ppm) were similar to those obtained with 1880 μg NO_2/m^3 (1 ppm) (Fig. 1). Both concentrations caused a statistically significant increase ($P < 0.05$) in PEN-induced S.T. of approx. 8 min after a single 3-h exposure. With 2 days of exposure, the magnitude of the increase in sleeping time was only 3.1 min; however, this increase was still judged statistically significant ($P < 0.05$). Following 3 days of exposure, differences appeared in that 9400 μg NO_2/m^3 (5 ppm) caused no significant change, whereas 1880 μg NO_2/m^3 (1 ppm) caused a significant decrease ($P < 0.05$) in PEN-induced S.T. of about 6 min. Concentrations of 940 μg NO_2/m^3 (0.5 ppm) for a 2 day exposure and 470 μg NO_2/m^3 (0.25 ppm) for a 1-day exposure resulted in significant increases ($P < 0.05$) in PEN-induced S.T. of 9 and 3.8 min, respectively. One or 2 days of exposure to 235 μg NO_2/m^3 (0.125 ppm) caused no significant alterations. For all experiments, there were no statistically significant effects of NO_2 on PEN-induced I.T. Over all experiments the PEN-induced I.T. for air exposed mice was 4.3 min and was 4.5 min for NO_2 exposed mice.

Since the magnitude of the effects diminished as the length of exposure increased, the 1880 μg NO_2/m^3 (1 ppm) study was extended to assess potential tolerance development. Some of the female mice which were exposed to 1880 $\mu g/m^3$ (1 ppm) for 4 days (3 h/day) were exposed to 9400 μg NO_2 (5 ppm) for 3 h on the fifth day. The pre-exposure appeared to have caused tolerance to the higher concentration of NO_2 since the observed PEN-induced S.T. of 20.4 min ± 0.7 SE (n = 45) was not statistically different ($P > 0.05$) from the 21.2 min ± 0.6 SE (n = 44) response for air-exposed mice.

DISCUSSION

Ambient concentrations of NO_2 (470 $\mu g/m^3$, 0.25 ppm) caused increased PEN-induced S.T. in female mice after a 3-h exposure. One or 2 days (3 h/day) exposure produced similar effects at 1880 and 9400 μg NO_2/m^3 (1 and 5 ppm), but at these higher concentrations, the effects appeared to be greater. At 940 μg NO_2/m^3 (0.5 ppm) the response was observed after the second daily 3-h exposure. It would appear that after the maximal increase in PEN-induced S.T. occurred, tachyphylaxis was observed. The mechanism for the observed tachyphylaxis and even the reversal of the effect as shown in the study at 1880 μg NO_2/m^3 (1 ppm), when 3 days (3 h/day) of exposure significantly decreased PEN-induced S.T., cannot be explained at this time. Tolerance in which exposure to a low concentration of NO_2

inhibits the effect of a subsequent exposure to a higher concentration of NO_2 has been described [21] and was observed in this study as well.

The primary mechanism for the termination of PEN action is aliphatic hydroxylation by the cytochrome P_{450}-, as opposed to cytochrome P_{448}-dependent microsomal mixed-function oxidase activity [1, 9]. The major metabolite is PEN alcohol [5-ethyl-5-(3′-hydroxy-1′-methylbutyl)barbituric acid] [3, 16]. The liver appears to be the predominant site of PEN metabolism as shown by Ossenberg et al. [15]. Thus, it is possible that NO_2 affected the duration of PEN narcosis by altering hepatic PEN metabolism. With the exception of an effect of NO_2 on lung prostaglandin metabolism [11] in rats and a description of increased sensitivity to carbachol caused by NO_2 exposure in humans [14] there are at present no other reports in the literature of effects of NO_2 at the low concentration used in this study (470 μg NO_2/m^3, 0.25 ppm, × 3 h).

Because of the nature of the oxidation in the lung initiated by NO_2, a number of reactive products are likely to be formed in the lung [10]. More recently, fatty acid epoxides have been detected in the lungs of rats exposed to NO_2 [18]. Mechanisms of transport of these products to the liver are speculative, but the potential for these events cannot be ignored, especially since distribution studies with labeled NO_2 have shown that the pollutant or reaction products are transported to extrapulmonary sites via the blood stream almost immediately after inhalation [7]. Bowden et al. [2] showed that 72 h after skin painting of mice with cholesterol-α-epoxide, the epoxide could be found in several organs including the liver.

Using identical techniques, PEN-induced S.T. studies have been conducted with O_3 [6, 8]. Concentrations as low as 196 μg O_3/m^3 (0.1 ppm) caused significant increases in PEN-induced S.T. in female CD-1 mice. Male mice were not affected. With O_3, as the concentration was decreased, it required an increasing number of daily 3-h exposures to observe the effect. For example, the maximal effect was detected at exposure day 2 at 1960 μg O_3/m^3 (1 ppm), and at day 16 at 196 μg O_3/m^3 (0.1 ppm). For NO_2, the effect was observed after the first exposure to concentrations as low as 470 $\mu g/m^3$ (0.25 ppm). Thus, for a single exposure, NO_2 is more effective than O_3. However with O_3 alterations in PEN-induced S.T. could be demonstrated at a lower concentration when the duration of intermittent exposure was increased. Tolerance to effects on PEN-induced S.T. was not observed after O_3 exposure [6], but it did occur upon NO_2 exposure, as reported here. From these comparisons, it appears that NO_2 and O_3 have different mechanisms of toxicity for xenobiotic pharmacokinetics. Thus, future studies will be conducted to examine the influence of NO_2 on mechanisms involved in PEN-induced S.T. to better elucidate toxicological differences between these two gaseous pollutants.

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